bio-protocol Preprint

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Respiration measurements

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An abbreviated version of this protocol was published in eLIFE in Oct 2019

Mitochondrial fusion is required for spermatogonial differentiation and meiosis

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Detailed protocol

Respiration measurements of isolated male germ cells

1. Testis digestion – modified from Gaysinskaya et al., 2014:

   a. Remove tunica albuginea, loosen seminiferous tubules, and place both testes in a 15 ml conical tube and add room temperature Dissociation Solution A.
   b. Shake in horizontal position at 150 rpm for 10 min at 35° C. Use same settings for all subsequent shaking steps, unless otherwise indicated.
   c. Halfway into the 10 min incubation, gently pipette solution up and down using disposable transfer pipets to help tubule dispersion.
   d. At the end of the 10 min incubation, gently pipette up and down a few more times. By the end of this step, tubules should be long and dispersed.

2. Somatic cell removal:

   a. Allow tubules to settle for 2 min at room temperature by standing the tube vertically.
   b. Remove and discard the supernatant enriched in interstitial cells, leaving just enough liquid to cover the settled tubules.

3. Seminiferous tubule digestion:

   a. Add 6 ml of Dissociation Solution B pre-heated to 37° C and gently pipette up and down 10 times. Shake for 25 minutes.
   b. Halfway into the 25 min digestion period add 60 µl of EDTA-Free 2.5 % Trypsin (Thermo Fisher; 15090046) and pipette the tubules again 10 times. At the end of the incubation time, re-pipette 10 more times. The tubules should be fragmented and solution dense with cells.

4. Preparation of cells for FACS:

   a. Pass the suspension through a 100 µm nylon cell strainer (Corning; 352340 BD).
   b. Pellet at 150 g for 5 min, remove supernatant, then add 5 µg/ml Hoechst 33342 for ploidy analysis. Stain for 30-45 min while shaking at 35° C at 50 rpm in the dark.
   c. Pellet at 150 g for 5 min, remove supernatant, then resuspend in 1 mL Flow Cytometry Buffer and filter through a 35 µm mesh into a 5 ml glass bottom tube (Falcon; 352235).
   d. If necessary, dilute cells to less than 20 million cells/ml.

5. FACS – modified from Bastos et al., 2005:

   a. Sort 2N, 4N, and 1N germ cells by their blue and red Hoechst fluorescence as described previously (Bastos et al., 2005).
   b. Sort cells directly into Germ Cell Collection Media.

6. Seahorse extracellular flux analysis:

   a. Pellet cells at 150 g for 5 min at 4°C then resuspend in Seahorse Media.
   b. Plate 50,000 cells per well of a XF96 cell culture microplate (Agilent; 102601-100), pre-coated with Cell-Tak (Corning; 354240) per manufacturer's instructions. Adjust final volume to 180 µl.
   c. Centrifuge the plate at 1,000 rpm for 5 min to promote cell adhesion.
   d. Incubate cells in Seahorse Media for 1 hr prior to performing the Seahorse analysis.
   e. Perform the Seahorse analysis using a Seahorse XF96 analyzer according to the manufacturer's instructions (Agilent; Seahorse XF Mitochondrial Stress Test Kit). The actual kit was not used. Injections were made into 180 ul of Seahorse media as follows:
      i. Port A: 20 ul of 50 µM Oligomycin

ii. Port B: 22 ul of 100 µM CCCP
iii. Port C: 25 ul of 50 µM Antimycin A
iv. Port D: empty; do not inject.

f. At the end of the experiment trypsinize and count the number of cells remaining in each well and use this information to normalize the OCR measurements.

Data analysis

a. Data analysis and normalization can be performed with the freely available Seahorse Wave Desktop Software.
b. Combine data from multiple experiments using the freely available XF Cell Mito Stress Test Report Generator.

Solutions and reagents

Dissociation Solution A

1. 6 ml HBSS with calcium, with magnesium, without phenol red (Thermo Fisher; 14025092)
2. Collagenase Type IV (Sigma; C5138) (200 U/ml or 1.6 mg/ml)
3. DNase I (Sigma; D5025)
   a. Make stock solution in PBS at 4 mg/ml, aliquot, and freeze.
   b. Use at 1:580 dilution
4. 6.6 mM sodium pyruvate
5. 0.05% sodium lactate (Sigma; L4263)

Dissociation Solution B

Dissociation Solution A with EDTA-free trypsin (Thermo Fisher; 15090046) added to 0.025%

Final Flow Cytometry Buffer

1. HBSS without phenol red, Ca2+, Mg2+, or EDTA (Thermo; 14175095)
2. 2.5 mg/ml fraction V BSA (Sigma; a4503)
3. 10 mM HEPES buffer
4. 6.6 mM sodium pyruvate
5. 0.05% sodium lactate (Sigma; L4263)
6. DNase I (50 ug/ml)
7. 1 mM MgCl2
8. pH to 7.1-7.2

Germ Cell Collection Media

1. DMEM (Thermo Fisher; 11995)
2. 10% FBS
3. 1% PS
4. 6.6 mM sodium pyruvate
5. 0.05% sodium lactate (Sigma; L4263)

Seahorse Media

1. DMEM (Sigma; D5030)
2. 5% FBS
3. 1% Pen/Strep
4. 2 mM glutamine
5. 6.6 mM sodium pyruvate
6. 0.05% sodium lactate (Sigma; L4263)
7. 10 mM glucose

References

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2. Gaysinskaya, V., Soh, I.Y., van der Heijden, G.W., and Bortvin, A. (2014). Optimized flow cytometry isolation of murine spermatocytes. Cytometry Part A : the journal of the International Society for Analytical Cytology 85, 556-565.

3. Varuzhanyan, G., Rojansky, R., Sweredoski, M.J., Graham, R.L., Hess, S., Ladinsky, M.S., and Chan, D.C. (2019). Mitochondrial fusion is required for spermatogonial differentiation and meiosis. eLife 8.

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1. Chan, D. C.(2019). Respiration measurements. Bio-protocol Preprint. bio-protocol.org/prep100.
2. Varuzhanyan, G., Rojansky, R., Sweredoski, M. J., Graham, R. L., Hess, S., Ladinsky, M. S. and Chan, D. C.(2019). Mitochondrial fusion is required for spermatogonial differentiation and meiosis. eLIFE. DOI: 10.7554/eLife.51601